Chemotropic responses by pearl millet pollen tubes

B.J. Reger, R. Chaubal*, and R. Pressey

USDA, ARS, Richard B. Russell Research Center, P.O. Box 5677, Athens, GA 30613, USA

Summary. The possible existence of a chemotropic factor controlling pearl millet pollen-tube directionality within the ovary was investigated using three approaches: cytochemical analysis of water-soluble components at the micropyle, in vitro testing of various chemicals for chemotropic activity, and an attempt to isolate and characterize an ovarian chemotropic factor. Observations of pollinated pearl millet ovaries by fluorescence microscopy revealed that pollen tubes enroute to the embryo sac exhibit specific directional turns in the regions of the style base/ovary juncture, the basal placenta, and the micropyle. The placenta and the micropylar regions have water-soluble periodic acid Schiff positive-substances as well as protein; this extracellular material and the walls of the micropylar nucellar cells appear to have associated calcium (Chaubal and Reger 1991). In vitro assays of pollen-tube behavior in response to a range of external stimuli revealed that pearl millet pollen tubes exhibit directional turns in response to: (1) diffusate from excised pistil tissues; (2) glucose, but not to several other carbohydrates; (3) calcium (unwashed polygalacturonic acid-calcium gel); (4) an ovarian water soluble, low molecular weight, acidic protein. These results which apparently suggest the presence of at least three different potential chemotropic factors in pearl millet ovaries are discussed in relation to angiosperm chemotropism in general and the difficulties involved in the search for possible in vivo chemotropic factors.

Key words: *Pennisetum glaucum* – Pollen-tube directionality – Chemotropism

Introduction

Fertilization in angiosperms involves sequential interactions of the male gametophyte (the pollen tube) with the female sporophyte (pistil) and the female gametophyte (embryo sac). The pollen tubes grow intercellularly

---

* On Specific Cooperative Agreement 58-43YK-8-0026 with the Department of Biochemistry, University of Georgia, Athens, GA 30602, USA

Offprint requests to: B.J. Reger

through the pistil tissues and enter the embryo sac with striking efficiency. Apparently, some control mechanisms operate within pistil tissues to maintain proper pollen-tube directionality. While mechanical (anatomical) (reviewed in Heslop-Harrison 1987; Heslop-Harrison et al. 1985; Heslop-Harrison and Reger 1988) and electrical (Marsh and Beams 1945; Zeijlemaker 1956; Sinyukhin and Baritikov 1967; Wang et al. 1989; reviewed in Robinson 1985) controls have been successfully investigated, attempts to identify chemotropic factors have been made by several investigators using a variety of techniques (Brink 1924; Tsao 1949; Linck and Blaydes 1960; Rosen 1961; Mascarenhas and Machlis 1962a, b, 1964; Miki-Hirosige 1964; Welk et al. 1965; Cook and Walden 1967; Glenk et al. 1971; Mulcahy and Mulcahy 1985, 1987; Hepher and Boulter 1987; Kandasamy and Kristen 1987; Sanders and Lord 1989; Robbertse et al. 1990); however, the identity of chemotropic factors not only remains elusive but their very existence in situ remains questionable.

In vitro testing of pollen-tube chemotropism to pistil parts and extracts and to various other substances has a long history (see reviews, Heslop-Harrison 1987; Mascarenhas 1978; Rosen 1968; Van Went and Willemse 1984; Vasil 1987). Initially, surveys were conducted to determine plant species whose pollen showed a chemotropic response to pistil parts: for example, 13 out of 39 plant species tested were positive, but of these 2 were responsive to other floral parts and to leaf tissue as well (Tsao 1949; Linck and Blaydes 1960). Surveys were followed by attempts to isolate chemotropic substances from pistil tissues (Tsao 1949; Mascarenhas and Machlis 1962b; Rosen 1961; Miki-Hirosige 1964). Although no chemotropic factor was isolated and identified, results with *Antirrhinum majus* suggested that the positive chemotropic factor was a small molecule, heat stable, water soluble, and associated with larger molecules (Mascarenhas and Machlis 1962b). These findings led to the search for inorganic ions and the discovery that calcium showed chemotropic activity for pollen of *Antirrhinum majus*, *Narcissus pseudonarcissus*, and *Clivia miniata* (Mascarenhas and Machlis 1964). However, pollen from three other genera, namely *Lilium* (Rosen 1964), *Oenothera* (Glenk et al. 1971), and *Zea* (Cook and Walden 1967), did not respond to calcium. Rosen (1971) therefore, concluded that calcium cannot be the universal chemotropic factor as originally suggested by Mascarenhas and Machlis (1964). Despite this apparent failure the micropyle continues to be a site where chemotropic control is invoked, as there is a lack of any mechanical control as well as the presence of secretions (Welk et al. 1965; Chao 1971), in most angiosperms to account for the abrupt turning of the pollen tubes toward the embryo sac.

Pollen-stigma interaction studies of normal and trichomeless pearl millet have revealed that the basal cell complex of the stigma trichome provides the initial guidance for pollen-tube entrance into the stigma axis to be in the direction of the ovary (Heslop-Harrison and Reger 1988). In the normal stigma, pollen tubes receiving their initial cue from the trichome base proceed linearly through the stigma and style to the ovary. In contrast, pollen tubes in the mutant (trichomeless) stigma, having missed the directional cue, grow randomly towards or away from the ovary, indicating the absence of an overriding chemotropic control in the stigma (Heslop-Harrison and Reger 1988). Whether mechanical, electrical, or chemotropic controls are operative in the ovary and providing directionality to pollen tubes remain to be determined.

Three observations in particular led us to investigate chemotropic controls in pearl millet ovaries. First, synergid cells of pearl millet and other angiosperms store the highest concentration of calcium relative to other cells of the ovary which, among other functions in sexual reproduction, may control pollen-tube directionality during the final stages of growth (Chaubal and Reger 1990a, b, 1991). Second, using pyroantimonate precipitation techniques it has been observed that in mature pearl millet ovaries calcium is probably present in low amounts along the pollen-tube pathway (appressed to the outer ovule integument and the micropyle). Third, pearl millet pollen tubes are abruptly arrested at the style base/ovary juncture in pistils 1–2 days post-optimum maturity, suggesting an accumulation of some chemical factor affecting pollen tube growth (Reger and Sprague 1983). Lower levels of this presumed chemical could exist in younger ovaries and influence pollen-tube directionality. Further, calcium could potentially accumulate at the style base/ovary juncture of older ovaries by diffusion from the ovary wall intercellular spaces, which also contain high levels of calcium (Chaubal and Reger 1991). Although these observations suggest the possible involvement of calcium in pollen-tube guidance, this element has been extensively tested by others (Mascarenhas and Machlis 1964; Rosen 1971; Glenk et al. 1971; Cook and Walden 1967) and found to be a chemotropic factor in vitro for some but not all pollens. We have undertaken a critical examination of chemotropic responses by pearl millet pollen tubes in vitro that we hope to correlate with in vivo and in situ data. In the study reported here we demonstrate the presence of extracellular periodic acid Schiff (PAS)-positive material and protein at the micropyle and in vitro chemotropic responses of pearl millet pollen tubes to glucose, calcium, and an ovarian protein.

Materials and methods

Plant material

Seed of male-fertile (Tift 23BE) and male-sterile (Tift 23AE) *Pennisetum glaucum* (L.) R. Br. [= *P. americanum* (L.) Leeke; *P. typhoides* (Burm.) Stapf et Hubb.] were supplied by Drs. G. Burton

and W. Hanna, USDA, ARS, Tifton, Georgia. The male-fertile and male-sterile plants were grown in separate greenhouses.

Fluorescence microscopy

To determine the pollen-tube pathway and possible sites of directional controls within the ovary in relation to the embryo sac, controlled pollinations of mature, Tift 23AE inflorescences were made using pollen from Tift 23BE. Pistils were harvested 6.5 h after pollination, sufficient time for the pollen tubes to have reached the embryo sac (Reger and James 1982), fixed in ethanol:-glacial acetic acid (3:1) for 2–3 h, cleared in 8 N NaOH for 18–24 h, stained in decolorized aniline blue (DAB, 0.1% aniline blue in 0.1 M K_3PO_4, pH 11), placed on microscope slides with a few drops of DAB, and viewed by epifluorescence microscopy using a Leitz Orthoplan with filter sets BP420–490, RKP510, LP515 and BP340–380, RKP400, LP430 (this set was useful in that autofluorescence was reduced greatly). Pollen tubes are localized within pistil tissues by the fluorescence of their inner callosic wall component following DAB staining (Linskens and Esser 1957). Pollen-tube directionality was noted in relation to the ovule, placenta, micropyle, and the embryo sac. Nonfluorescence microscopy was also used. Finally, pistils were gently squashed using an 18 × 18 mm cover glass for improved viewing and photography of the pollen tubes at the micropyle.

Cytochemistry

For the staining of PAS-positive substances and protein, mature unpollinated ovaries from Tift 23AE were fixed by being plunged into liquid propane at −189 to −191° C and then dried by substitution with anhydrous acetone containing 3.5% glutaraldehyde (Chaubal and Reger 1990a, 1991). After substitution, samples were washed with anhydrous acetone (five changes, 5 min each), postfixed with 2% OsO_4 in anhydrous acetone for 2 h at room temperature, washed with anhydrous acetone (three changes, 5 min each), and embedded in Spurr's Resin. Thick sections (0.25–1.0 μm) were cut using a Reichert MT2B ultramicrotome and a diamond histoknife. Sections were collected on acetone-cleaned glass slides and heated gently (40° C, 4 min) to allow the sections to adhere to glass. To stain for PAS-positive substances, sections were incubated with 1% aqueous periodic acid for 1 h in a humid chamber, washed with distilled water for 5 min, and incubated with Schiff's reagent (Sigma) for 6–9 h. To stain for protein, sections were treated with 1% Amido Black 10B (C.I. 20495) in 7% acetic acid. In control treatments, incubation of the sections with periodic acid prior to Schiff's staining was either omitted or replaced with a distilled water incubation. Additionally, PAS and Amido Black stainings were also performed with sections of chemically-fixed (3.5% glutaraldehyde, 2% OsO_4) ovaries.

Pollen and pollen bioassay

For pollen collection, inflorescences of Tift 23BE undergoing anthesis were excised, cleaned of exerted anthers and stigmas, transferred to water, and brought into the laboratory. Pollen-shed was promoted by exposure to a 300-W floodlamp. Pollen was collected from newly exerted and dehiscing anthers and placed in a humid atmosphere – a square Petri dish with two moistened Kimwipes and two microscope slides upon which was placed a square of aluminum foil containing the pollen. The pollen was allowed to hydrate for 1 h before being used in experiments, and was always used within 3 h of collection.

Semi-solid media were used throughout these studies. Media consisted of 10% sucrose (Schwarz/Mann ultra pure), 1 mM H_3BO_3, and either 1% agarose [FMC SeaKem (LE), lot no. 70818]

or 1% IsoGel [FMC agarose, lot no. 62130]. Assays were conducted on microscope slides layered with 0.5 ml solidified medium. Slides were prepared by adding 0.5 ml molten medium to the slide and placing a cover glass (no. 1, 24×40 mm) on top. Prepared slides were stored in humid boxes for use throughout the day. For testing, the cover glass was removed, a filter paper disc (Whatman #1, 3 mm diameter) soaked with 1.5 µl test solution was placed on the medium, pollen was applied in lines at various distances from the disc using a single, camel-hair bristle brush, and the test slide was placed in a humid box. Generally, lines of pollen were placed at 1, 2, 3, and 4 mm from the disc either on the same slide or on two different slides. Test slides were examined after 1 h and later. Such a microscope slide set-up, where diffusion of the test material from the disc is fairly rapid, appeared to be suitable for pearl millet pollen because of its fast germination (ca. 5 min) and growth (ca. 0.5 mm/h).

Characteristically, pearl millet pollen placed in a line exhibit tube elongation equally in both directions from the line, thus having a "bottle-brush" appearance when observed with a dissecting microscope (Fig. 5). When pollen tubes respond chemotropically, pollen-tube extension appears essentially unidirectional because pollen tubes extending in the opposite direction from the chemotropic factor turn and grow toward the active factor (Figs. 4, 6A–C, 7A–C). It was thus possible to visually assess the response of pollen tubes to the test material without making counts of the pollen tubes. Pollen grains placed singly as opposed to line placement on the agarose displayed the same turning phenomenon (Fig. 6C).

Pollen-tube response to pistil-tissue diffusate

To test pollen-tube response to pistil parts, freshly harvested pistils were separated into stigmas, styles, and ovaries (cut in half longitudinally) by cross-sectional cuts at the base of the stigma and the base of the style and assayed immediately on the agarose medium. Pollen was placed in lines 1 mm from each pistil part. Pollen was also placed appropriately to determine any preferential responses, e.g., top versus base of style. Sliced ovary halves were placed cut side down on the agarose, and lines of pollen were applied on all slides of each half. Pollen-tube directionality was recorded following 1–2 h of incubation in humid boxes.

Pollen-tube response to carbohydrates and amino acids

Carbohydrates were tested for chemotropic activity on the basis of both the strong chemotropic response to all pistil-tissue exudates and our previously unpublished data on stigma carbohydrate composition showing abundant free glucose (Table 1) and positive Yariv staining of all cut pistil tissues, which is indicative of arabinogalactans. Pectins were included because pollen tubes grow intercellularly within the pistil in an environment of pectic polysaccharides. Also, pollen and pollen tubes are known to contain and secrete pectinase (Heslop-Harrison 1979; Pressey and Reger 1989).

Monosaccharides tested included: glucose, 1 mM to 1 M; arabinose, galactose, fructose, fucose, mannose, and xylose at 1,5 mM and 1 M.

Polysaccharides tested were arbinogalactan (larchwood) at 5 and 50 mg/ml and β-glucan (barley) at 1 mg/ml and the dry chemical (without using a disc). In addition, pectin solutions were tested; they included apple pectin (Sigma P-2157) and citrus fruit pectin (Sigma P-9135), both of which were without sucrose and other sugars, and de-esterified polygalacturonic acid (PG) (Sigma P-1879). Pectin solutions were 5 mg/ml with pH adjusted to 5.5, and they were tested at 1 and 5 mg/ml.

Some amino acids were tested because leucine and methyltryptophan were reported to be chemotropically active for *Oenothera* (Schildknecht and Benoni 1963) and because free asparagine is abundant in pearl millet stigmas (Evans et al. 1987).

The amino acids tested were asparagine, 2% (pH adjusted to 5.6); proline, 2%; leucine, 1%; tryptophan, 1%.

Carbohydrate composition of stigmas

Approximately 4 g fresh weight of stigmas were harvested, ground thoroughly with dH₂O in a mortar and pestle, and centrifuged for 15 min at 39000 g. The supernatant was divided into two equal portions, and one was dialyzed against dH₂O. Following dialysis, both samples were lyophilized. The samples were hydrolyzed by dissolving in 2 N trifluoroacetic acid and heating at 121° C for 1 h. The free monosaccharides were then reduced and acetylated according to the method of York et al. 1985. The peracetylated alditols were analyzed by gas chromatography using a Supelco 2340 column.

Pollen-tube response to calcium

Considerable attention was given to an appropriate in vitro test of calcium as a possible chemotropic factor for pearl millet pollen tubes. Inherent problems exist because some calcium in the medium is required for pollen germination and pollen-tube growth (Brewbaker and Kwack 1963, 1964); however, this can confound any response of pollen tubes to an externally applied calcium gradient. To reduce the level of calcium contamination in the medium, we used IsoGel [FMC agarose, lot. no. 62130; 139 µg Ca/g agar] instead of agarose [FMC Seakem (LE), lot. no. 70918; 736 µg Ca/g agar]. The calcium concentration of these agars were determined from ashed samples, 0.5 g each, with a Jarrell-Ash Model Atom-Comp direct-reading inductively coupled argon plasma (ICAP) spectrometer. These agars were also tested for biologically significant calcium contamination by observing pollen behavior on them with and without calcium supplementation. Thus, a 1 mM Ca(NO₃)₂ supplementation of the agarose medium, which is optimal for pearl millet pollen germination and pollen-tube growth in liquid medium, resulted in early tube arrest, indicative of too much calcium, whereas pollen-tube growth was enhanced on 1 mM Ca(NO₃)₂-supplemented IsoGel medium. Pearl millet pollen display the population effect (Brewbaker and Kwack 1963). The germination of singly placed pollen grains on IsoGel medium was rare; therefore, lines of pollen were used in all of the bioassays.

Solutions of Ca(NO₃)₂ and CaCl₂ ranging from 1 mM to 1 M were tested using 1.5 µl per disc; however, results were inconsistent, indicating that gradients may not have been properly established. Dry-powder forms of Ca(OH)₂ and CaCO₃, and pectin-calcium gel were tested without the use of discs. The pectin-calcium gel was prepared by mixing thoroughly 1 ml 5 mg/ml de-esterified PG (pH adjusted to 5.5) and CaCl₂ or Ca(NO₃)₂ (e.g., 50 µl 1 M CaCl₂ for a final concentration of 50 mM) in a 1.5-ml microfuge tube and centrifuging the mixture in a Beckman microfuge B (maximum 8733 g) to pellet the gel. The supernatant was discarded, and a small portion of the unwashed gel was removed with a spatula, carefully blotted of excess solution, and placed on the IsoGel slide for testing. The controls included CaCl₂ and Ca(NO₃)₂ (1 mM to 1 M), 1 and 5 mg/ml of the stock PG, and dH₂O, 1.5 µl applied to the test discs; pectin-calcium gel washed 3 times with dH₂O before testing; unwashed pectin-calcium gel tested on Isogel medium supplemented with 1 mM CaCl₂ or Ca(NO₃)₂.

Tissue extracts and separation

Pistil tissues were harvested with the aid of a dissecting microscope. Following lemma and palea removal from each floret the pistil was subdivided into stigma, style, and ovary. Pistil tissues (i.e., stigmas, styles, and ovaries) were kept on ice during collection and then were either used immediately or frozen in liquid N₂ and stored at −80° C for use later.

Prior to separation of tissue water-extracts by ion-exchange chromatography, water-extracts were obtained as described below, and the following fractions were tested for chemotropic activity: homogenate pellets and supernatants; supernatant fractions smaller than 10 kDa, larger than 10 kDa, and heat-treated ones (10 min in boiling H_2O-bath) larger than 10 kDa. Generally, the non-heat-treated fractions larger than 10 kDa and the homogenate pellets were active chemotropically (some positive evidence for the fraction smaller than 10 kDa with high dilution). Homogenate pellets remained positive even after two H_2O washes, 1 M NaCl wash followed by two H_2O washes, and 0.2 M $CaCl_2$ wash followed by two H_2O washes. Fractions larger than 10 kDa on IEF gel electrophoresis (described below) revealed ovarian proteins to be acidic; therefore, further fractionation by ion exchange was performed using a Mono Q anion column.

Water-extracts were obtained from collected tissues (stigmas, styles, and/or ovaries) using glass homogenizers. Homogenates were centrifuged for 15 min at 39 000 g. The supernatants were desalted and concentrated using Centricons, 10 000 MW cutoff. Supernatants were fractionated by ion exchange using a Mono Q (anion; 0.02 M NaOAc, pH 6.0 and 0–1.0 M NaCl) column and a fast protein LC Pharmacia System. Fractions were collected and assayed for chemotropic activity. Active fractions were desalted, concentrated, and characterized electrophoretically. Where appropriate, harvested tissue homogenates, supernatants, and ion-exchange fractions were kept at 0°–2° C. Protein was determined by the method of Lowry et al. (1951). The Pharmacia Phast System was used to run Native-PAGE Phast Gel 8 to 25 gradient gels and Phast Gel IEF 3 to 9 gels. Gels were fixed and stained with Coomassie and silver according to the Pharmacia Phast System instructions. Identical Native-PAGE gels were also fixed and stained with Stains-all according to Campbell et al. (1983).

Carbohydrate analysis of the active fraction as determined by bioassay and flanking inactive fractions of an ovarian preparation separated using the Mono Q column was performed as described earlier.

Based on the results of these experiments (positive chemotropic response to potential calcium-binding ovarian protein, possibly glycoprotein, and persistent pollen-tube attraction to homogenate pellets), the following proteins were tested for chemotropic activity: calmodulin (1.7 mg/ml $\pm$ 1 mM Ca^{++}), ovalbumin (1 mg/ml), carbonic anhydrase (1 mg/ml), crude glucose oxidase (1 mg/ml), myoglobin (1 mg/ml), and peroxidases (horseradish) (1 mg/ml) Sigma P-1014 type VIII (acidic), Sigma P-1139 type IX (basic), and Sigma P-8375 type VI: 2 (basic).

Results

In vivo pollen-tube pathway

Observations by fluorescence microscopy revealed that pollen tubes entering the ovary appear to splay out and grow downward over the outer ovule integument, between the inner ovary wall and the outer ovule integument, toward the rudimentary basal placenta (Figs. 1, 2A, B). Pollen tubes appear to avoid growing in the direction that would lead to a path between the inner ovary wall and the incomplete ovule integuments, which appears to be the shortest route to the micropyle and embryo sac (Figs. 1, 2A). Once the placental region is reached, pollen tubes turn (Fig. 2B) and continue their passage between the placenta and outer ovule integument to the micropyle, where they turn and grow intercellularly through the nucellus to the embryo sac (Fig. 2C).

Cytochemistry

In median longitudinal sections of pearl millet ovaries, the micropyle appears as an opening a few cell layers below and lateral to the embryo sac. Ovule integuments extending from the micropylar end of the ovary are prominent at the micropyle. Chalazally extending integuments do not reach the micropyle (Fig. 1). In freeze-fixed ovaries treated with the PAS reagent, a dense red staining extracellular material appressed to surfaces of the inner and outer ovule integuments in the micropylar region was observed (Fig. 3A, B). Some PAS-positive extracellular material was also localized along the outer ovule integument extending from the micropylar end (Fig. 3A). The filiform apparatus also stained strongly with PAS (Fig. 3C). In some sections a few nucellar cells between the micropyle and the filiform apparatus were faintly stained and apparently degenerated. No staining was observed in sections treated with the PAS reagent without prior periodic acid incubation. In unstained as well as PAS-stained sections the extracellular material at the micropyle and appressed to the ovule integument, as well as the filiform apparatus stained dense blue when stained with Amido Black 10B (Fig. 3A–C), indicating the presence of protein. In chemically fixed ovaries the extracellular material was not observed, apparently due to its loss during sample preparation; the filiform apparatus stained faintly with both PAS and Amido Black 10B.

Pollen-tube response to pistil parts, carbohydrates, and amino acids

Strong, positive chemotropic responses were observed by pearl millet pollen tubes to all pistil-part diffusates


Fig. 1. Diagram of a pollinated pearl millet ovary. *A* Antipodals, *CC* central cell, *E* egg cell, *II* inner integument of the ovule, *M* micropyle, *OI* outer integument of the ovule, *OL* ovary locule, *OW* ovary wall, *P* placenta region, *S* synergid cell, *PT* pollen tube. Note pollen-tube path in relation to the embryo sac

Fig. 2A–C. Fluorescence microscopy of pollen tubes in an ovary 6.5 h after pollination. **A** Orientation of the ovary and fluorescing pollen tubes are as diagrammed in Fig. 1. The *arrowhead* indicates the location of the egg apparatus. $\times$ 60. **B** Same ovary as seen in Fig. 2A turned 90° to view fluorescing pollen tubes from placental side. Abaxial view in relation to the embryo sac. Note pollen tubes in the placental region turn toward the micropyle (*arrows*). $\times$ 60. **C** Same ovary as seen in Fig. 2A squashed gently to show turning of a pollen tube at the micropyle. $\times$ 160

Fig. 3A–C. Longitudinal sections of freeze-substitution fixed pearl millet ovaries treated with the PAS reagent followed by Amino Black 10B staining for protein. **A** Note staining of the extracellular material appressed to the ovule integuments in the micropylar region (*arrowheads*). The PAS-positive material and protein are also present in the filiform apparatus (*arrowhead*). $\times$ 60. **B** Higher magnification of the PAS-positive and protein extracellular material at the micropyle (*M*). The extracellular material occurs closely appressed to the outer ovule integument. $\times$ 60. **C** Higher magnification of the egg apparatus showing PAS staining of the filiform apparatus (*FA*). $\times$ 239
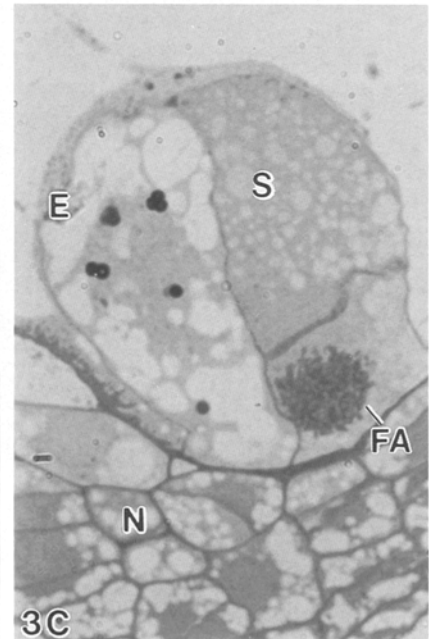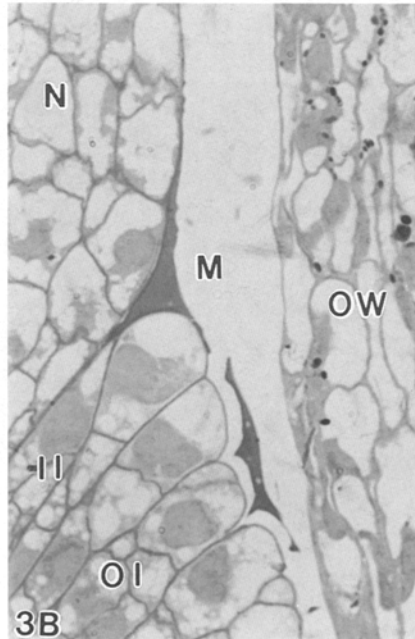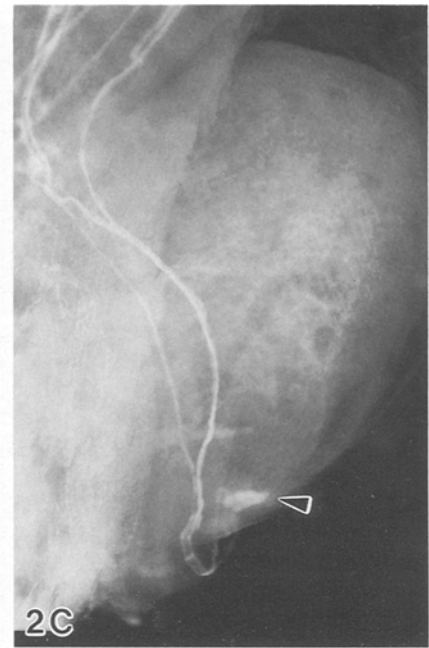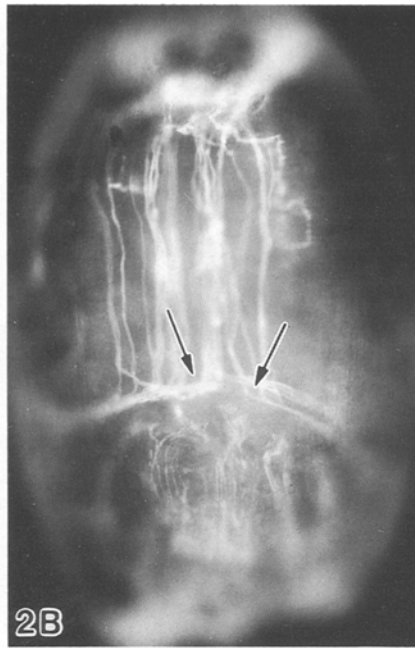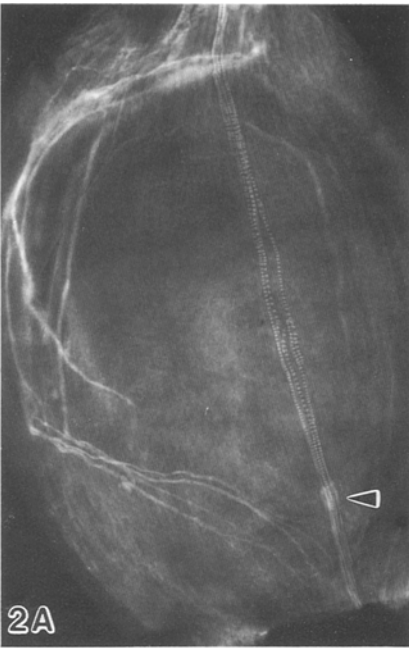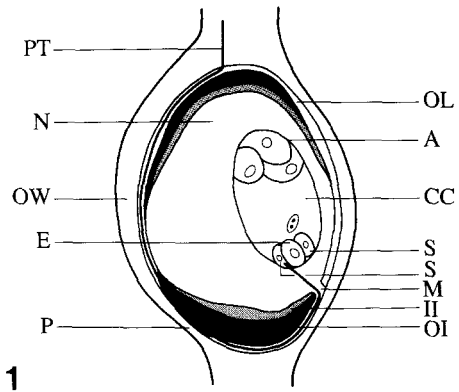
Figs. 1–3
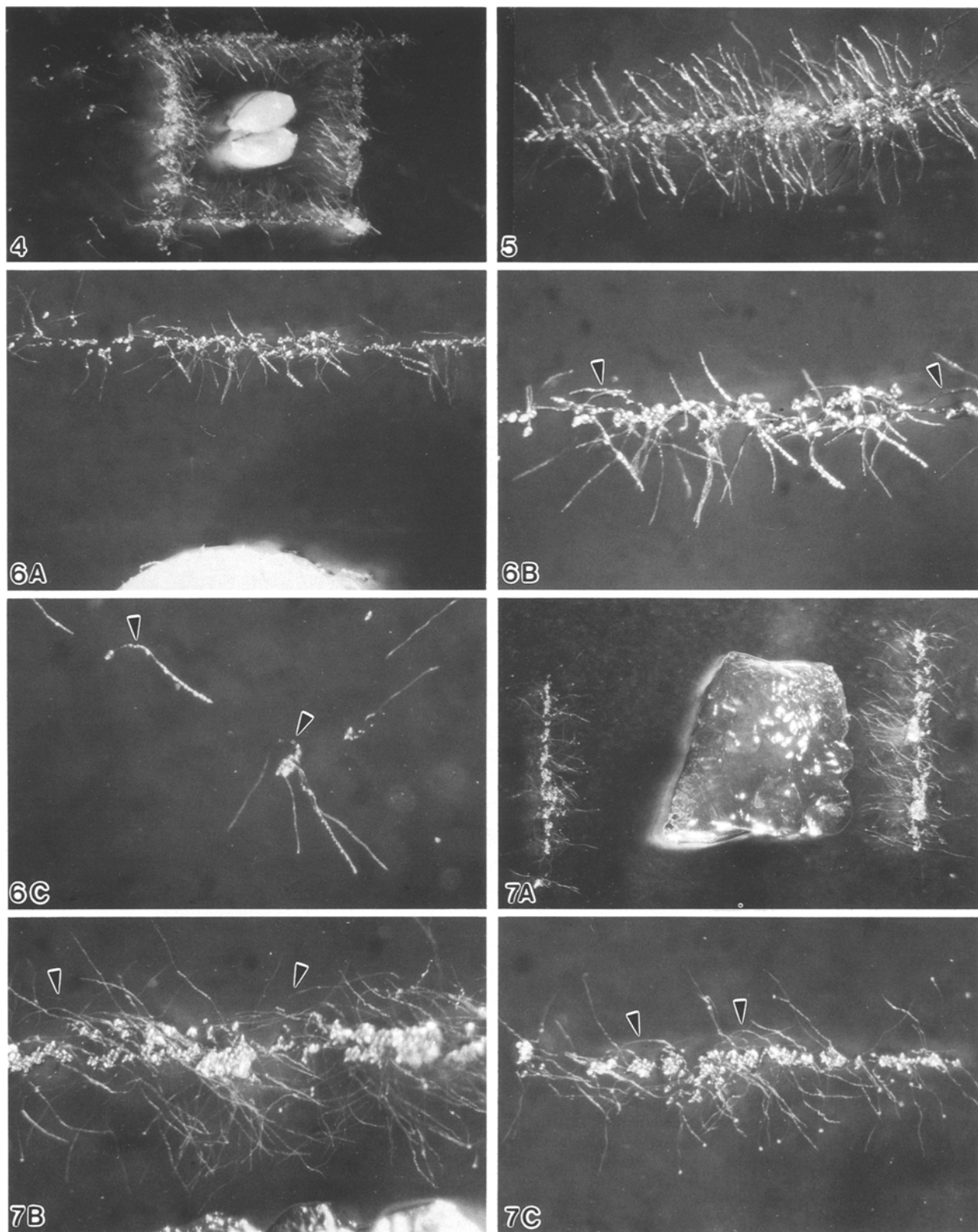
Fig. 4. Chemotropism by pearl millet pollen tubes to diffusate from a sliced pearl millet ovary. Note that pollen-tube growth is in the direction of the ovary tissue. × 8. Similar responses were observed for diffusates from stigma and style tissues. **Fig. 5.** Pearl millet pollen displaying bidirectional growth from line-placed pollen. Note that in this control treatment tube growth (ca. 3 h) is equal in both directions from line of pollen. × 12. **Fig. 6A–C.** Positive chemotropic response by pearl millet pollen tubes (1 h) to glucose. **A** Disc with 1.5 µl 1 *M* glucose and line of pollen 2 mm from the disc. × 14. **B** Higher magnification of pollen showing unidirectional pollen-tube growth pattern. The *arrowheads* indicate pollen

tubes that have turned toward the disc. × 24. **C** Clump of four pollen grains 3 mm from the disc. Note extreme turn (*arrowhead*) by one tube in the clump. Singly placed grain in upper left corner, approximately 4 mm from the disc, also shows turning (*arrowhead*). × 35. **Fig. 7A–C.** Positive chemotropic response by pearl millet pollen tubes (2 h) to pectin-calcium gel. **A** Pectin-calcium gel with lines of pollen placed 1 and 2 mm from gel. Note the unidirectional pollen-tube growth pattern toward the pectin-calcium gel. × 8. **B** Higher magnification of 1-mm pollen line. *Arrowheads* indicate turned pollen tubes. × 18. **C** Higher magnification of the 2-mm pollen line. Note turned pollen tubes (*arrowheads*). × 17

Table 1. Carbohydrate composition of pearl millet stigmas

Monosaccharide	Non-dialyzed	Dialyzed μg/g fresh weight	Free
Rhamnose	13.34	6.14	7.20
Arabinose	149.74	126.16	23.58
Xylose	88.20	41.02	47.18
Mannose	90.26	55.38	34.88
Galactose	210.26	112.80	97.46
Glucose	1448.20	85.00	1364.10

and to glucose (100 mM and 1 M/disc) (Fig. 6A–C). Only bidirectional tube growth (Fig. 5) occurred when tests were conducted using arabinose, galactose, fructose, fucose, mannose, xylose, arabinogalactan, β-glucan, apple pectin, citrus fruit pectin, de-esterified PG, asparagine, proline, leucine, and tryptophan. Pearl millet stigmas contain arabinogalactans and very high amounts of free glucose (Table 1). One millimolar glucose supplemented to the agarose medium greatly diminished pollen-tube response to pistil-part diffusates, i.e., there was more bidirectional than unidirectional pollen-tube growth.

Pollen-tube response to calcium

Pearl millet pollen tubes responded chemotropically positive to unwashed pectin-calcium gel (Fig. 7A–C). We suspect that this substance provided a slow release of adhering calcium, which set up an appropriate gradient on the IsoGel medium for pollen-tube response. No chemotropic response was observed when the IsoGel medium was supplemented with 1 mM $CaCl_2$ or $Ca(NO_3)_2$, the pectin-calcium gel was washed thoroughly with dH_2O, or when the pectin solutions were tested.

Tissue extracts and separation

Generally, chemotropic activity was observed with stigma, style, and ovary water-extracts of fractions larger than 10 kDa, and activity was lost after boiling. Pellets resulting from the centrifugation of tissue homogenates of all pistil parts remained very active chemotropically, and this activity was not lost with water and salt washes. Fractions smaller than 10 kDa were inhibitory unless very dilute.

IEF gel electrophoresis of stigma, style, and ovary water-extracts from 400 pistils showed the style and ovary to have mainly acidic proteins, while the stigmas were found to contain both acidic and basic proteins (Fig. 10A). Figure 8 illustrates the Mono Q separation profiles of fractions larger than 10 kDa from water-extracts of stigmas, styles, and ovaries (dissection and subdivision of 400 pistils). Different sensitivity scales were used because of major differences in protein concentration between the stigma, style, and ovary. Ovaries have approximately 6 times and 13 times more protein than stigmas and styles, respectively. Chemotropic activity
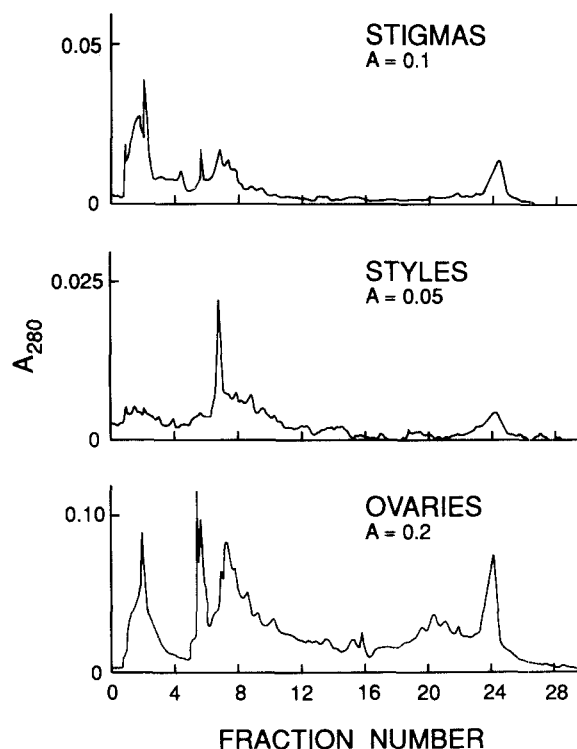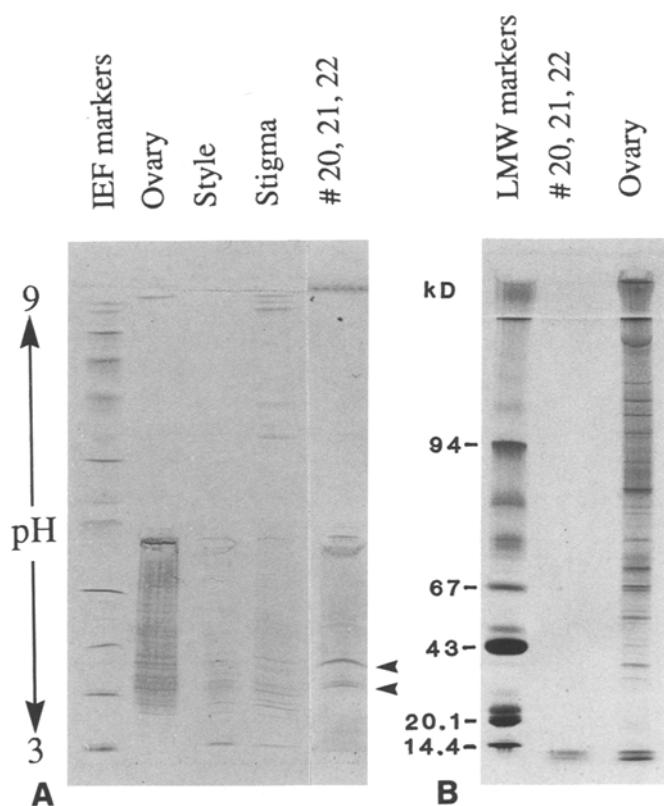

Fig. 8. Profiles of water-extracts of 400 pearl millet pistils (subdivided into stigmas, styles, and ovaries) separated using Mono Q anion exchange column chromatography
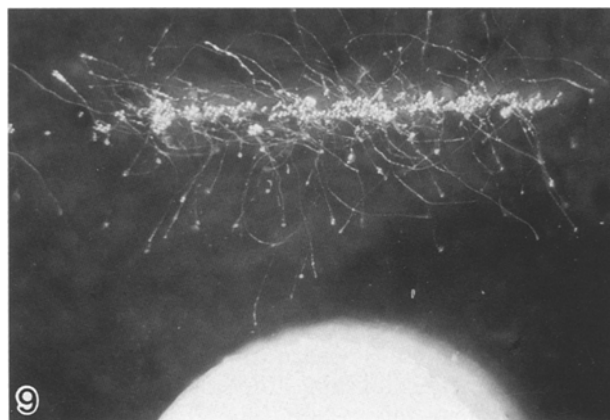
Fig. 9. Positive chemotropic response of pearl millet pollen tubes to fraction 21 of a pearl millet ovarian water-extract separated using Mono Q anion column chromatography (see Fig. 8). All other fractions were chemotropically inactive. ×16

was observed in fraction number 21 (Fig. 9) in this and other preparations. Ovarian fractions number 20, 21, and 22 were combined, dialyzed, concentrated and analyzed electrophoretically. Native-PAGE of an aliquot of the ovarian preparation larger than 10 kDa prior to separation on Mono Q and the Mono-Q-separated, active fraction revealed the active fraction to be of low MW; this low MW fraction was also present in the initial preparation (Fig. 10B). The lowest MW marker protein was 14.4 kDa, and the active fraction (two bands) was slightly less. This fraction also stained blue with Stains-

IEF markers
Ovary
Style
Stigma
20, 21, 22
LMW markers
20, 21, 22
Ovary

9
pH
3
A

kD
94—
67—
43—
20.1—
14.4—
B

Fig. 10 A, B. Electrophoretic characterization of tissue water extracts and ovarian chemotropically active fraction (no. 21 and flanking tubes; see Fig. 8). **A** Coomassie blue-stained IEF markers and water extracts of the 400 stigmas, styles, and ovaries prior to Mono Q separation and the silver-stained ovarian, chemotropically active fraction following Mono Q separation. Note major band, approximately pI 4.9, and minor band, approximately pI 4.3. **B** Silver-stained native-PAGE of Pharmacia low molecular weight markers, ovarian water extract prior to Mono Q anion separation; and ovarian, Mono Q separated, chemotropically active fraction. The molecular weight of the chemotropically active fraction is less than the lowest molecular weight marker of 14.4 kDa

all, indicating that it may be a calcium-binding protein. However, more protein is needed to run the appropriate controls for firmly establishing this since heavily phosphorylated proteins also stain blue (Campbell et al. 1983). IEF of the active fraction revealed a major band with a pI of approximately 4.9 and a minor band with a pI of approximately 4.3 (Fig. 10A). Carbohydrate analysis of an ovarian, active Mono Q fraction equivalent to fractions number 20, 21, and 22 and an inactive fraction equivalent to number 24 showed very little carbohydrate. Of the low amount of carbohydrate present, the active fraction contained 6.5% xylose, 21.9% mannose, and 71.6% glucose, and the inactive fraction contained 17.8% xylose and 82.1% glucose. The carbohydrate composition of these fractions may be indicative of glycoprotein.

No chemotropic response was observed to the following proteins: calmodulin $\pm$ Ca^{++}, ovalbumin, carbonic anhydrase, crude glucose oxidase, myoglobin, and acidic and basic peroxidases.

Discussion

In the ovary of pearl millet sites of pollen-tube directional control as evidenced by fluorescence microscopy are possible at the top of the ovary, at the placental region, and at the micropyle. In conjunction with the known function of stigma trichomes to mechanically direct pollen tubes toward the ovary (Heslop-Harrison and Reger 1988), this indicates that in pearl millet pollen tubes reach the micropyle by responding to signals at different strategic locations. The propensity of pollen tubes to remain in the raphal region and the lack of resolvable ovule integuments in the chalazal region suggest that pollen tubes may perceive another mechanical cue from the ridged epidermis of the raphe, as suggested for *Zea* (Heslop-Harrison et al. 1985), and thus be guided from the top to the base of ovary. However, detailed cytological investigation is needed to determine this. Once the pollen tubes reach the placenta their turning toward the micropyle may be either mechanical (following the ovary locule) or chemical (secretions). The turning of the pollen tube at the micropyle toward the embryo sac is not obviously mechanical, and as such is another potential site for chemotropic control in pearl millet, as well as in most angiosperms. The cytochemical data presented here confirm in pearl millet the earlier findings in *Paspalum* (Chao 1971, 1977) regarding the presence of these extracellular water-soluble substances, PAS positive and protein, in the vicinity of the micropyle. In pearl millet ovaries the location of these substances and associated low amounts of calcium (Chaubal and Reger 1991) precisely corresponding to the pollen-tube pathway from the placental region to the micropyle may be indicative of their possible involvement in controlling pollen-tube directionality in this region.

In vitro studies demonstrated that pearl millet pollen tubes respond chemotropically positive to pistil-part diffusates, glucose, calcium, and an ovarian protein. The positive response of pearl millet pollen tubes to diffusates of all pistil parts is probably due to abundant amounts of free glucose since supplementing the agarose medium with glucose diminished the chemotropic response. However, based on our observation of a persistent positive response of the pollen tubes to washed tissue homogenates, other factors could be involved. Although some calcium must also be present in the diffusates, it is doubtful that the observed response was to it as the tests were conducted on agarose rather than IsoGel.

Despite the use of relatively calcium-free media and the achievement of good germination (90%) by line placement of pollen, the positive response by pearl millet pollen to externally supplied calcium salts and solutions at various concentrations and distances appeared difficult to reproduce consistently except when unwashed pectin-calcium gel was used. It seems that only the pectin-calcium gel established a calcium gradient that was compatible with the germination time and elongation rate of pearl millet pollen. Through a comparison of our results with reports on a positive (*A. majus*) and

lack of chemotropic response (*Lilium* and *Zea*) to calcium, another limiting factor in the establishment of an appropriate gradient becomes evident. The calcium requirements for the germination and tube elongation of a pollen may be different. If the calcium requirement for pollen-tube elongation is either similar to or less than that required for germination, the calcium needed to obtain germination (supplied externally or through the phenomenon of the population effect) will have already surpassed the level required for an appropriate gradient of calcium to which a pollen tube can respond. In *A. majus* (Mascarenhas and Machlis 1964), the exiting of pollen tubes growing within a pollen clump in response to external calcium suggests that their calcium requirement for tube elongation may not have been optimally fulfilled in the clump. In contrast, the exiting of *Lilium* pollen tubes without any externally supplied calcium (Welk et al. 1965) and the ability of singly placed pollen grains to germinate and elongate normally (Rosen 1971) suggests that the calcium requirements for *Lilium* pollen germination and tube elongation are not different. In *Zea* the optimum calcium requirement for germination is 300 ppm (2.7 mM) $CaCl_2$; its calcium requirement for tube extension is lower than this because a few pollen grains that germinated at 50 ppm (0.45 mM) $CaCl_2$ exhibited normal pollen-tube growth rates, and on aqueous media pollen tubes grew into regions that did not support pollen germination (Cook and Walden 1967). In pearl millet the high germination of line-placed pollen and the bidirectional elongation of pollen tubes in both low calcium-contaminated medium (IsoGel) and on agarose without calcium supplementation, and the positive response to an apparently appropriate calcium gradient established through the use of pectin-calcium gel suggests that the optimal calcium requirement for tube elongation is slightly higher than that required for germination. Such a narrow difference between calcium requirements for pollen germination and tube elongation in pearl millet would make the establishment of appropriate gradients in vitro difficult.

Pollen-tube growth has been shown to occur by tip elongation resulting from the fusion of polysaccharide particles (Heslop-Harrison and Heslop-Harrison 1982), and data from in vitro studies have shown the control of these processes by optimum levels of calcium (Picton and Steer 1982, 1983, 1985; Steer and Steer 1989); thus, directional changes of a pollen tube could occur by something such as calcium momentarily affecting tip growth (Mascarenhas 1978). While it is tempting to think that calcium may be involved in the pollen tube turning at the micropyle, based on our knowledge of the above and that high concentrations of calcium are stored in synergids, the presence of calcium in adjacent nucellar cell walls (Chaubal and Reger 1991), and the in vitro demonstration of calcium as a chemotropic agent, we have no direct evidence as yet to support this hypothesis. At least two other chemotropic factors, glucose and the ovarian protein, must be considered as well. Determining how these additional factors affect pollen-tube directionality and their location in situ in conjunc-

tion with the proposed cryo studies for calcium (Chaubal and Reger 1991) should lead to an understanding of this commonly observed phenomenon.

This is the first demonstration of positive chemotropism by a pistil protein, specifically an ovarian protein. It is water soluble, has a very low molecular weight (less than 14.4 kDa), and is very acidic (ca. pI 4.9). Further characterization of this protein is underway, including studies to establish with certainty preliminary indications that it is a highly phosphorylated glycoprotein and/or a calcium-binding glycoprotein.

This study makes apparent a few major limitations in the overall understanding of chemotropism in pearl millet ovaries. Although glucose, calcium, and an ovarian protein have been identified as being chemotropically positive through in vitro bioassays, some additional factors, possibly present in homogenate pellets, may remain to be identified. In situ localization of all these factors, particularly free water-soluble glucose and calcium, may not always be possible. While there is an indication of how calcium affects tip growth and pollen-tube directionality in vitro, this remains to be determined for glucose and the ovarian protein. Finally, considering indirect information such as the co-localization of calcium with PAS-positive substances and protein in vivo, the possible calcium-binding properties of the ovarian protein, and perhaps even the effect of free glucose on potassium and calcium channels as in insulin-producing cells (Henquin 1987; Meats et al. 1989; Satin and Cook 1985) necessitates investigations to determine whether these factors operate interactively.

References

Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50:859–865

Brewbaker JL, Kwack BH (1964) The calcium ion and substances influencing pollen growth. In: Linskens HF (ed) Pollen physiology and fertilization. North-Holland, Amsterdam, pp 143–151

Brink RA (1924) The physiology of pollen IV. chemotropism; effects on growth of grouping grains; formation and function of callose plugs; summary and conclusions. Am J Bot 11:417–436

Campbell KP, MacLennan DH, Jorgensen AO (1983) Staining of the Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with cationic carbocyanine dye "Stains-all." J Biol Chem 258:11267–11273

Chao C (1971) A periodic acid-Schiff's substance related to the directional growth of pollen tube into embryo sac in *Paspalum* ovules. Am J Bot 58:649–654

Chao C (1977) Further cytological studies of a periodic acid-Schiff's substance in the ovules of *Paspalum orbiculare* and *P. longifolium*. Am J Bot 64:921–930

Chaubal R, Reger BJ (1990a) Relatively high calcium is localized in synergid cells of wheat ovaries. Sex Plant Reprod 3:98–102

Chaubal R, Reger BJ (1990b) Demonstration of high calcium levels in synergid cells of angiosperm ovaries. In: Leonard RT, Hepler PK (eds) 13th Annu Symp in Plant Physiol "Calcium in Plant Growth and Development". Am Soc Plant Physiol, Rockville, Md., pp 193–194

Chaubal R, Reger BJ (1992) Calcium in the synergid cells and other regions of pearl millet ovaries. Sex Plant Reprod 5:34–46

Cook FS, Walden DB (1967) The male gametophyte of *Zea mays* L. III. The influence of temperature and calcium on pollen germination and tube growth. Can J Bot 45:605–613

Evans JJ, Pressey R, Reger BJ (1987) Amino acids in stigmas of *Pennisetum americanum*. Phytochemistry 26:2661–2663

Glenk HO, Wagner W, Schimmer O (1971) Can Ca^{++} ions act as a chemotropic factor in *Oenothera* fertilization? In: Heslop-Harrison J (ed) Pollen development and physiology. Butterworths, London, pp 255–261

Henquin JC (1987) Regulation of insulin release by ionic and electrical events in β cells. Horm Res 27:168–178

Hepher A, Boulter ME (1987) Pollen tube growth and fertilization efficiency in *Salpiglossis sinuata*: implications for the involvement of chemotropic factors. Ann Bot 60:595–601

Heslop-Harrison J (1979) Aspects of the structure, cytochemistry and germination of the pollen of rye (*Secale cereale* L.). Ann Bot [Suppl 1] 44:1–47

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. Int Rev Cytol 107:1–78

Heslop-Harrison J, Heslop-Harrison Y (1982) The growth of the grass pollen tube: 1. Characteristics of the polysaccharide particles ("P-particles") associated with apical growth. Protoplasma 112:71–80

Heslop-Harrison Y, Reger BJ (1988) Tissue organization, pollen receptivity and pollen tube guidance in normal and mutant stigmas of the grass *Pennisetum typhoides* (Burm.) Stapf et Hubb. Sex Plant Reprod 1:182–193

Heslop-Harrison Y, Heslop-Harrison J, Reger BJ (1985) The Pollen-stigma interaction in the grasses. 7. Pollen-tube guidance and the regulation of tube number in *Zea mays* L. Acta Bot Neerl 34:193–221

Kandasamy MK, Kristen U (1987) Pollen tube growth in the style of *Nicotiana sylvestris* is neither influenced by the ovary nor directed by a gradient. J Plant Physiol 131:495–500

Linck AJ, Blaydes GW (1960) Demonstration of the chemotropism of pollen tubes in vitro in four plant species. Ohio J Sci 60:274–278

Linskens HF, Esser K (1957) Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepropfen nach Selbstung und Fremdung. Naturwissenschaften 33:16

Lowry OJ, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Marsh G, Beams HW (1945) The orientation of pollen tubes of *Vinca* in the electric current. J Cell Physiol 25:195–204

Mascarenhas JP (1978) Sexual chemotaxis and chemotropism in plants. In: Hazelbauer GL (ed) Receptors and recognition. Chapman and Hall, London, pp 171–203

Mascarenhas JP, Machlis L (1962a) Chemotropic response of *Antirrhinum majus* pollen to calcium. Nature 196:292–293

Mascarenhas JP, Machlis L (1962b) The pollen-tube chemotropic factor from *Antirrhinum majus*: bioassay, extraction, and partial purification. Am J Bot 49:482–489

Mascarenhas JP, Machlis L (1964) Chemotropic response of the pollen of *Antirrhinum majus* to calcium. Plant Physiol 39:70–77

Meats JE, Best L, Lynch AM, Tomlinson S (1989) Glucose increases cytosolic calcium concentration and inositol lipid metabolism in HIT-T15 cells. Cell Calcium 10:534–541

Miki-Hirosige H (1964) Tropism of pollen tubes to the pistil. In: Linskens HF (ed) Pollen physiology and fertilization. North-Holland, Amsterdam, pp 15–158

Mulcahy GB, Mulcahy DL (1985) Ovarian influence on pollen tube growth, as indicated by the semivivo technique. Am J Bot 72:1078–1080

Mulcahy GB, Mulcahy DL (1987) Induced pollen tube directionality. Am J Bot 74:1458–1459

Picton JM, Steer MW (1982) A model for the mechanism of tip extension in pollen tubes. J Theor Biol 98:15–20

Picton JM, Steer MW (1983) Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. Protoplasma 115:11–17

Picton JM, Steer MW (1985) The effects of ruthenium red, lanthanum, fluorescein isothiocyanate and trifluoperazine on vesicle transport, vesicle fusion and tip extension in pollen tubes. Planta 163:20–26

Pressey R, Reger BJ (1989) Polygalacturonase in pollen from corn and other grasses. Plant Sci 59:57–62

Reger BJ, James J (1982) Pollen germination and pollen tube growth of sorghum when crossed to maize and pearl millet. Crop Sci 22:140–144

Reger BJ, Sprague JJ (1983) Pearl millet and sorghum pollen tube growth in pearl millet gynoecia of different ages. Crop Sci 23:931–934

Robbertse PJ, Lock JJ, Stoffberg E, Coetzer LA (1990) Effect of boron on directionality of pollen tube growth in *Petunia* and *Agapanthus*. S Afr J Bot 56:487–492

Robinson KR (1985) The responses of cells to electrical fields: a review. J Cell Biol 101:2023–2027

Rosen WG (1961) Studies on pollen-tube chemotropism. Am J Bot 48:889–895

Rosen WG (1964) Chemotropism and fine structure of pollen tubes. In: Linskens HF (ed) Pollen physiology and fertilization. North-Holland, Amsterdam, pp 159–166

Sanders LC, Lord EM (1989) Directed movement of latex particles in the gynoecia of three species of flowering plants. Science 243:1606–1608

Satin LS, Cook DL (1985) Voltage-gated Ca^{2+} current in pancreatic β-cells. Pflügers Arch 404:385–387

Schildknecht H, Benoni H (1963) Über die Chemie der Anziehung von Pollenschläuchen durch die Samenanlagen von Oenotheren. Z Naturforsch 18 B:45–54

Sinyukhin AM, Britikov EA (1967) Action potentials in the reproductive system of plants. Nature 21:1278–1280

Steer MW, Steer JM (1989) Tanksley Review No. 16. Pollen tube tip growth. New Phytol 111:323–358

Tsao TH (1949) A study of chemotropism of pollen tubes in vitro. Plant Physiol 24:294–504

Van Went JL, Willemse MTM (1984) Fertilization. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 273–317

Vasil IK (1987) Physiology and culture of pollen. In: Giles KL, Prakash J (eds) Pollen cytology and development. Academic Press, New York London, pp 127–174

Wang C, Rathore KS, Robinson KR (1989) The responses of pollen to applied electrical fields. Dev Biol 136:405–410

Welk M, Millington WF, Rosen WG (1965) Chemotropic activity and the pathway of the pollen tube in lily. Am J Bot 52:774–781

York WE, Darvill AG, McNeil M, Stevenson TT, Albersheim P (1985) Isolation and characterization of plant cell walls and cell-wall components. Methods Enzymol 118:3–40

Zeijlemaker FCJ (1956) Growth of pollen tubes in vitro and their reaction on potential differences. Acta Bot Neerl 5:179–186